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Evaluating potential biomarkers of cachexia and survival in skeletal muscle of upper gastrointestinal cancer patients

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Short title: Biomarkers of cancer cachexia

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Keywords: Cachexia, Cancer, Biomarker, Skeletal muscle

Abbreviations used: BMI, body mass index; COPD, chronic obstructive pulmonary disease; CRP. C-reactive protein; DGC, dystrophin glycoprotein complex; HC, healthy control; KPS, Karnofsky performance score; MyHC, myosin heavy-chain; qRT-PCR,

real-time quantitative reverse transcription polymerase chain reaction; SD, standard deviation; UGIC, upper gastrointestinal cancer; UPP, ubiquitin proteasome pathway.

Article category: Cancer therapy

Impact and novelty: There remains an unmet clinical need for therapeutic targets and diagnostic biomarkers in cancer cachexia. The current study is the largest investigation to date to systematically analyse potential skeletal muscle biomarkers of cancer cachexia related to weight-loss and survival in humans. Akt protein levels/phosphorylation status and GABARAPL1 expression are identified as novel biomarkers relating to cancer/early cachexia, β-dystroglycan as a biomarker of weight-loss and myosin heavy-chain and dystrophin as biomarkers associated with survival.

Abstract

In order to grow the potential therapeutic armamentarium in the cachexia domain of supportive oncology there is a pressing need to develop suitable biomarkers and potential drug targets. This pilot study evaluated several potential candidate biomarkers in skeletal muscle biopsies from a cohort of upper gastrointestinal cancer (UGIC) patients. 107 patients (15 weight-stable healthy controls (HC), 92 UGIC patients) were recruited. Mean (SD) weight-loss of UGIC patients was 8.1 (9.3)%. Cachexia was defined as weight-loss ≥5%. *Rectus abdominis* muscle was obtained at surgery and analysed by Western blotting or qRT-PCR for Akt/phosphorylated-Akt (n=52), FOXO1/3a, MAFBx, MuRF1, BNIP3, GABARAPL1 (n=59), myosin heavy-chain (MyHC, n=54), dystrophin (n=39), β-dystroglycan and β-sarcoglycan (n=52). Patients were followed up for an average of 1255 days (range 581-1955 days) or until death. Cancer patients compared with HC, had reduced total Akt protein (p=0.001), increased ratio of phosphorylated to total Akt (p=0.002) and increased expression of GABARAPL1 (p=0.024). β-dystroglycan levels were higher in cachectic compared with non-cachectic cancer patients (p=0.007). Survival was shortened in patients with low MyHC levels or low dystrophin levels (p=0.023 and p=0.008 respectively). The present study has identified intramuscular protein level of β-dystroglycan as a potential biomarker of cancer cachexia. Changes in the structural elements of muscle (MyHC or dystrophin) appear to be survival biomarkers.

Introduction

Cancer cachexia represents an important, yet often under-appreciated cause of patient morbidity and mortality. It is "a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment" (1). Cachexia is due to a combination of reduced food intake and metabolic change. The prevalence of cachexia varies with tumour type and stage and may also vary with the genotype of the host (2). Affected individuals face increased risks of treatment failure (be it chemotherapy, radiotherapy or surgery), increased risks of treatment side-effects and an increased mortality rate (1;3).

Cachexia in its advanced phase (where patients may have lost 20-30% of their body weight) is easily identified, but by this stage, it is often impossible to undertake any realistic form of multimodal rehabilitation. Thus, it might be useful to identify patients who are at risk or in the early phase of cancer cachexia so that targeted intervention can be instituted. An early intervention approach has been hampered by a limited understanding of the molecular pathways implicated in human cancer cachexia along with a lack of validated biomarkers. For example, although elevated serum C-reactive protein (CRP) is a robust indicator of systemic inflammation and has been linked to both cancer-associated hypermetabolism, reduced food intake and shortened survival (4), in a multivariate model of weight-loss in upper gastrointestinal patients, the estimate of size of effect on degree of weight-loss for CRP was only 34% (5). Loss of skeletal muscle has been identified as the central characteristic of cancer cachexia (1). We

hypothesised that either alteration in pathways of muscle atrophy or in the components of muscle itself might provide more robust biomarkers.

Muscle wasting occurs as a result of an imbalance between protein synthesis and degradation. Evidence from animal models of muscle atrophy suggests that the catabolic ubiquitin proteasome pathway (UPP) and autophagy pathway are of key importance (6-10). Despite this knowledge, there remains limited data relating to human cancer cachexia. A few studies have identified potential biomarkers of cachexia in various tissue compartments using different methodological approaches (2;11-14). As a useful addition to this emerging body of work, we sought to identify potential clinically relevant cachectic biomarkers in skeletal muscle biopsies from upper gastrointestinal cancer (UGIC) patients in relation to weight-loss and post-operative survival. Candidate markers were selected according to previous literature and included Akt and phosphorylated Akt (pAkt), FOXO transcription factors, ubiquitin E3 ligases (control of muscle anabolism/catabolism) (6-8;15;16), BNIP3 and GABARAPL1 (as markers of autophagy) (6;9;17;18), myosin heavy-chain (MyHC), dystrophin, β-dystroglycan and β-sarcoglycan (as markers of structural alteration in muscle) (7;10;19;20).

Materials and Methods

Subjects

92 consecutive UGIC patients (with a diagnosis of oesophago-gastric (n=56), small bowel (n=2), pancreatic (n=33) or common bile duct (n=1) malignancy) undergoing potentially curative surgery were recruited. 16 patients had stage IV disease, 38 patients had stage III, 23 patients had stage II and 15 patients had stage I disease. Patients (n=27) who had completed a course of neoadjuvant chemotherapy had not received chemotherapy in the four weeks prior to surgery/biopsy. No subjects were knowingly taking anabolic/catabolic agents, had uncontrolled diabetes or thyroid disorders. The weight-stable healthy controls (HC) comprised 15 subjects undergoing abdominal surgery for non-malignant, non-inflammatory conditions. Written informed consent was obtained from all subjects and ethical approval received from Lothian Research Ethics Committee (UK). UGIC patients were followed up for an average of 1255 days (range 581-1955 days) post-operatively or until death.

Anthropometry, weight-loss and performance status

Body weight was measured in light clothing using a beam scale (Seca, UK). Height was measured using a standard wall-mounted measure. The patients' clinical details were recorded and degree of weight-loss from self reported pre-illness (~6 months previously) stable weight documented. Patients were classified as cachectic if they had weight-loss ≥5% according to the modern definition (1). Karnofsky performance score (KPS) was assessed in each patient by a single observer.

Muscle biopsies

All biopsies were taken at the start of open abdominal surgery under general anaesthesia. Patients had undergone an overnight fast. The edge of the *Rectus abdominis* was exposed and a $1cm^3$ specimen removed using sharp dissection. Tissue samples were quickly cleaned of blood, flash frozen in liquid nitrogen and stored at -80°C until further analysis.

Blood measures

All blood samples were taken following an overnight fast. CRP level was measured using ELISA (Ely, UK). A CRP ≥5mg/l (the upper limit of normal in our lab) was considered consistent with the presence of systemic inflammation.

Protein Isolation

Approximately 20mg of muscle was homogenised in 0.5ml of lysis buffer (Triton – X100 (1%), NaCl (150mM), Tris-HCl (50mM), EDTA (1mM), PMSF (1mM), protease inhibitors (Roche Diagnostics) (1 tablet per 10ml), water to 10ml) using a Powergen 125 (Fisher Scientific) electric homogeniser. Samples were left on ice for 15min prior to centrifuging at 13000rpm for 15min. The supernatant was removed, and protein concentration was determined by comparing equal volumes of sample solution to known standards using the Lowry method. Samples were then stored at -80°C.

Nuclear protein extraction (for FOXO transcription factors)

Approximately 20mg of muscle was resuspended in 180µl of low salt lysis buffer (10mM HEPES, 10mM KCl, 1.5mM MgCl2, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, protease inhibitors (Roche Diagnostics) (1 tablet per 10ml)) and

ground using a hand held homogeniser. Samples were incubated on ice for 5min before two cycles of freeze-thaw lysis. After a brief vortex, samples were centrifuged at 4000rpm for 3min. The supernatant was removed and the pellet (containing the nuclei) resuspended in 40µl high salt extraction buffer (20mM HEPES, 420mM NaCl, 1mM EDTA, 1mM EGTA, 25% Glycerol, 1mM DTT, protease inhibitors (Roche Diagnostics) (1 tablet per 10ml)). Samples were incubated on ice for 30min with gentle mixing of the tubes every 5-10min. Samples were centrifuged at 4000rpm for 5min at 4°C. The supernatant which now contained the nuclear proteins was aliquoted into tubes and stored immediately at -80°C.

Western Blotting

20µg of protein from each sample was added to 3µl of 4 x LBS (0.5M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.05% β-mercaptoethanol, 0.004% bromophenol blue) and boiled for 3 minutes. Proteins were resolved using SDS-polyacrylamide gel electrophoresis at 160V for 45 mins. Proteins were transferred to a nitrocellulose membrane (80mA for 1 hour) using semi-dry transfer (Biorad). Membranes were blocked with either 3% BSA/TBST (TBS, 0.05% Tween) overnight at 4°C or with 5% milk/TBST for 1hr at room temp. Incubation with primary antibody (1:1000) was carried out in either 3% BSA/TBST or 0.5% milk/TBST solution at room temperature for 2 hours or overnight at 4^{o}C. Membranes were washed with TBST and primary antibody binding detected using horseradish-peroxidase conjugated secondary antibodies (1:2000 to 1:5000). Specific signal was detected using ECL (enhanced chemiluminescence) reagent (GE Healthcare) and exposure on photographic film (Kodak). Films were scanned and densitometry values estimated using ImageJ (NIH)

software. All proteins were normalised to alpha-skeletal actin as a loading control, except for FOXO1/3a which, because they were nuclear protein extracts, were normalised to lamin A/C.

Antibodies

The primary antibodies used in the study were Akt, pAkt (Ser473), FOXO1, FOXO3a (Cell Signaling); lamin A/C (Santa Cruz Biotechnology Inc); dystrophin (MANDYS102 (7D2)), β-dystroglycan (MANDAG2 (7D11)), (Developmental Studies Hybridoma Bank); β-sarcoglycan (abcam); myosin heavy chain (fast) (Sigma); alpha-skeletal actin (Novocaestra). Secondary antibodies were anti-mouse or anti-rabbit: (Upstate).

RNA Isolation

Total RNA was extracted from approximately 20mg of muscle using TRIzol (Invitrogen) reagent according to the manufacturer's directions. The RNA pellet was resuspended in DEPC treated water and RNA concentration was determined using a Nanodrop spectrophotometer (LabTech International, UK). RNA quality was assessed using 260/280, 230/260 ratios and the RIN score from the BioAnalyzer 2100 instrument (Agilent Technologies).

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted as described above. cDNA was prepared using 1μg RNA, TaqMan reverse transcription reagents (Applied Biosystems) and random hexamer primers (Applied Biosystems). Primers were designed to span introns using Primer Express 3.0 software (Applied Biosystems) and the primers were constructed by

Invitrogen (Paisley, UK). Primer sequences used were: BNIP3_Fw; *GTC AAG TCG GCC GGA AAA TA*, BNIP3_Rv; *GCG CTT CGG GTG TTT AAA GA*, GABARAPL1_Fw; *CCA CCG CAA GGA GAC AGA AG*, GABARAPL1_Rv; *GAA AAT GTG ATG ACG GTG TGT GT*, MAFBX_Fw; *CCG GCT GTT GGA GCT GAT A*, MAFBX_Rv; *TTG GGC GAT GCC ACT CA*, MURF1_Fw; *GCT AGG CGT GGC TCT CAT TC*, MURF1_Rv; *TCC TGG ATC AGG CTC GAC TT*. Samples were run on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems) in triplicates of 20 microlitres per well using SYBR Green PCR Master Mix (Applied Biosystems) as per manufacturer's instructions. Expression levels were normalised to ribosomal 18S RNA and results examined using the delta-delta CT method (21).

Statistical analysis

For analysis of qRT-PCR and Western blotting results, SPSSv19.0 was used. Mathematical transformation was performed when appropriate. Student's two tailed t test, Mann Whitney or Kruskal Wallis test were used to compare means between groups. Contingency tables were constructed where relevant and analysed by Chi squared test. Patients were divided into those who survived more than or less than one year post-operatively (a meaningful surgical and oncological outcome). ROC analysis was performed using this division and the cut-off which gave the highest sensitivity and specificity was manually selected. Kaplan-Meier survival curves and log-rank comparison was used to assess differences in survival between groups. Statistical significance was set at a p-value (two-tailed) of ≤ 0.05.

Results

107 patients were recruited in total (15 HC and 92 UGIC patients). Demographics for the entire cohort are illustrated in **Table 1**. Biopsies were used according to availability of tissue for different biomarkers. There were four separate groups and the demographics for these are illustrated in **Supplementary Table 1. Supplementary Figure 1** shows the overlap between groups. No significant differences were evident between these groups and the entire cohort.

Compared with HC, cancer patients were older (mean (SD) age 65 (10) vs 56 (17) years, p=0.003), had higher average weight-loss (8.1 (9.3) vs 0 (0) %, p=0.001), lower BMI (25.7 (4.0) vs 28.0 (4.5) kg/m^2, p=0.046) and a significantly lower KPS (89 (13) vs 100 (0), p=0.001) (**Table 1A**).

Within the cancer patient cohort, cachectic patients compared with non-cachectic patients had a larger proportion of females (19/51 vs 7/41, Chi squared p=0.033), were younger (63 (9) vs 68 (9) years, p=0.022), had shortened median survival (562 vs 846 days, p=0.030), and a lower BMI (24.6 (3.7) vs 27.0 (4.0) kg/m^2, p=0.004. KPS was also significantly lower in cachectic compared with non-cachectic patients (86 (14) vs 92 (10), p=0.020) (**Table 1A**).

Variability of protein biomarkers in the presence of cancer and cachexia

Results of the skeletal muscle biomarkers are illustrated in **Figure 1**. Level of total Akt protein was reduced in cancer patients compared with HC (0.49 (0.31) vs 0.89 (0.17), p=0.001), but there was no significant difference in pAkt protein level (0.47 (0.34) vs

0.29 (0.2), p=0.104). However, the ratio of pAkt to total Akt (indicative of Akt activity) was increased in cancer patients compared with HC (1.33 (1.04) vs 0.32 (0.21), p=0.002).

Cachectic cancer patients had significantly higher levels of β-dystroglycan than non-cachectic cancer patients (1.01 (0.16) vs 0.87 (0.20), p=0.007). There was also a trend towards increased levels of β-sarcoglycan (0.63 (0.28) vs 0.55 (0.55), p=0.052).

Variability of mRNA biomarkers in the presence of cancer and cachexia

Results of the skeletal muscle biomarkers are illustrated in **Figure 1**. There was a trend towards an increase in expression of BNIP3 in cancer patients compared with HC (1.37 (0.49) vs 1.07 (0.57), p=0.058 and a significantly increased expression of GABARAPL1 (1.60 (0.76) vs 1.10 (0.57), p=0.024). No mRNA biomarkers related to the presence of cachexia.

Variability of biomarkers associated with survival

Patients who survived ≤1 year post-operatively compared with those who survived >1 year had significantly higher average weight-loss (12.0 (11.1) vs 6.3 (8.0) %, p=0.007) and a lower KPS (83 (13) vs 91 (12), p=0.004) (**Table 1B**).

Given that there are no 'normal' cut-offs for skeletal muscle protein levels or mRNA expression for potential markers, ROC analysis was performed. There were no strong significant candidates, but there was a trend for MyHC, dystrophin and pAkt (area under the curves were 0.674 (p=0.069), 0.714 (p=0.070) and 0.669 (p=0.068)

respectively). Co-ordinates of the ROC curves were inspected and a cut-off of ≥0.87 chosen for MyHC to give a sensitivity of 84.8% and specificity of 46.2%; a cut-off of ≥0.31 chosen for dystrophin to give a sensitivity of 85.0% and specificity of 55.6%; and a cut-off of ≥0.19 chosen for pAkt to give a sensitivity of 87.5% and specificity of 42.3%. Kaplan-Meier survival analysis using these cut-offs (**Figure 2**) showed a significantly shorter survival for those with lower compared with higher MyHC levels (median survival 316 vs 1326 days, p=0.023) and lower compared with higher dystrophin levels (median survival 341 vs 660 days, p=0.008), but no significant difference between high and low pAkt levels (p=0.320). Given the difference in survival for MyHC and dystrophin, the demographics of the low versus higher levels of MyHC and dystrophin groups were examined (**Table 2**). None of the variables differed significantly between the groups for either MyHC or dystrophin.

Discussion

In this biomarker discovery study, we demonstrated suppression of total Akt protein levels in the skeletal muscle of cancer patients but with a relative increase in Akt activity. There was also some evidence of increased autophagy in cancer patients. β-dystroglycan appeared to relate to the presence of significant weight-loss in cancer patients. Low MyHC and low dystrophin protein levels both related to shortened survival.

In one of very few similar studies, Schmitt et al examined protein levels and phosphorylation status of muscle atrophy/hypertrophy pathway components in eight pancreatic cancer patients with cachexia compared with eight weight-stable cancer or pancreatitis patients (22). They observed reduced levels of Akt, MyHC and FOXO1 in the cachectic group. In the current study, which looked at a much larger cohort of patients with a variety of upper gastrointestinal cancers, along with non-cancer HC, we did not observe any differences in these markers between cachectic and non-cachectic patients. We did, however, observe cancer patients (compared with non-cancer HC) to have a reduction in Akt levels, but with a relative increase in overall Akt activity (expressed as the ratio of pAkt to total Akt). It should be noted that whilst FOXO3 antibody and methodology for determining MyHC levels differed, the antibodies for FOXO1, Akt and pAkt were the same between the two studies and would thus not explain these contrasting results. An alternative is that the observations of Schmitt et al may be tumour specific or reflect markers of more moderate cachexia whereas our observations relate to the presence of cancer alone or to cachexia earlier in the disease process. Schmitt et al defined cachexia as >10% weight-loss in 6 months whereas for

the current study, cachexia was defined as ≥5% weight-loss in line with recent definitions (1). However, when we analysed our data according to a 10% weight-loss cut-off, the results for individual variables did not differ from using a 5% weight-loss cut-off (data not shown). Therefore, another explanation would be that total protein turnover is suppressed in cancer patients (23) with a reduction in the available pool of Akt and that increased phosphorylation represents a compensatory mechanism. In support of this, in COPD patients with cachexia, an increased ratio of pAkt to total Akt has also been observed with the suggestion that this represents an attempt to restore muscle mass (24;25).

The muscle-specific E3 ubiquitin ligases, MuRF-1 and atrogin-1/MAFbx are commonly used to indicate activation of the UPP after the discovery that they were upregulated in several distinct models of atrophy (6). Likewise, markers of autophagy have been shown to be increased in cachectic mice and under the control of FOXO3 (17). However, in the current study, FOXO transcription factors and the ubiquitin E3 ligases were similar between HC and cancer patients and were not influenced by the presence of cachexia. Cancer patients did have increased expression of GABARAPL1 and a trend towards increased expression of BNIP3, both of which play a key role in autophagy. However, we did not see either of these autophagy markers significantly relating to survival or weight-loss. There was evidence of increased β-dystroglycan protein levels in cachectic patients and a trend towards increased protein levels of β-sarcoglycan. Dysregulation of the dystrophin glycoprotein complex (DGC) is a feature of muscular dystrophies and has been associated with cachexia (19). In the context of muscular dystrophy there would normally be downregulation of all components of the

DGC. However, this has not been the case in cancer cachexia but neither has an increase in protein levels been demonstrated (19). In a mouse model of muscular dystrophy treated with an AMPK activator, increased utrophin coincided with an increase in β-dystroglycan and resultant strengthening of the sarcolemma (26). It is therefore conceivable that the relationship between β-dystroglycan and cachexia seen in the current study represents an attempt at muscle membrane repair as it enters a more dysregulated state with progressive weight-loss. Given that the current study is not mechanistic and utrophin was not investigated as a potential marker, this suggestion is speculative.

It is striking that we demonstrated an association between low levels of structural muscle proteins and shortened survival. The lack of significant demographic differences between the low and higher level MyHC/dystrophin groups suggests that they are bona fide markers of post-operative survival. Perhaps lower levels of these structural proteins identify a susceptible population where muscle structure/membrane integrity has already started to become compromised. Alterations in membrane structure and integrity have been demonstrated in C-26 tumour bearing mice which is thought to be due, at least in part, to disruption of the DGC (19). This normally provides a strong mechanical link between the intracellular cytoskeleton and extracellular matrix (27). Mutations in the DGC cause muscular dystrophies/ cardiomyopathy, and a link with human cancer cachexia has been made. DGC deregulation was demonstrated in oesophago-gastric cancer patients and related to the presence of significant weight-loss (>10%) and systemic inflammation and to a shortened survival (19). It is also thought that there is selective targeting of

myofibrillar proteins, in particular MyHC, in cancer cachexia (7;10). In addition, myofibrillar degradation appears to occur in a time dependent manner. One animal study of muscle atrophy after denervation or fasting, demonstrated early targeting of thin filament components with subsequent loss of MyHC (20). Adding to the concept that membrane damage is important in the pathogenesis of cancer cachexia, our lab has recently shown that the presence of various myosin species in the urine of patients with oesophago-gastric cancer relates to significant (>10%) weight-loss (11). Our observations in the current study that patients with lower skeletal muscle dystrophin or MyHC protein levels are associated with a shortened survival are entirely consistent with this concept. Therefore, measurements of these structural elements in skeletal muscle appear to be suitable biomarkers relating to survival in UGIC cancer patients.

By comparing cachectic, non-cachectic and HC groups, this study evaluates the potential of certain variables to act as biomarkers of cachexia. However, it is not possible to determine the precise role of these variables in cachexia by comparing the cachectic with non-cachectic groups. The patients without cachexia at diagnosis represent a mixed group some of whom will remain weight-stable, but a significant other group will progress to cachexia and are therefore in a pre-cachectic state. This heterogeneity within the weight-stable group potentially masked changes in some variables which may play a role in development of cachexia/pre-cachexia. In order to further explore this area, longitudinal assessments of patients would be required to determine which individuals progress to losing weight after the initial biopsy. However, the natural history of cachexia in this study population cannot be determined because the majority of patients subsequently underwent a potentially curative resection.

It is important to appreciate that this study is capturing a snapshot in time of what is really a journey comprising early, cachectic and refractory phases (1) in a heterogenous population at various points on this spectrum. There is evidence that different proteolytic/synthetic pathways may be activated or repressed according to degree of weight-loss. For example, in a study of lung cancer patients with mean weight-loss of 2.9%, the lysosomal but not the UPP was activated (28), whereas in patients with gastric cancer and mean weight-losses of 5.2% and 5.6% have shown increases in components of the UPP (29;30). There is also evidence in cancer patients (31) that UPP activity increases with weight-loss up to 12-19% and then declines as disease severity progresses. Likewise, a recent study of gastric cancer patients showed evidence of increase in calpains in patients with minimal or no weight-loss, but did not show any difference in expression of the ubiquitin E3 ligases in cancer compared with control patients (32). Longitudinal studies in human cancer cachexia may be informative in this regard, but are difficult to carry out owing to the requirements for multiple assessments/ tissue samples in a frail population. In addition, there may be varying responses among patients to adjuvant chemoradiotherapy, the influence of surgery, post-operative complications, concurrent illnesses (eg infections) and selective attrition which will add to the complexity of interpreting such studies.

Non-cachectic patients were 5 years older than cachectic patients. Sarcopenia of ageing has been well characterised and the non-cachectic group would thus have 5 extra years of age-related muscle wasting. It is possible that this age gap may have influenced differences in levels of biomarkers between groups. Conversely, a younger age at

diagnosis of cancer is a risk factor for poorer outcome in certain tumour types (eg breast (33)). Whether younger patients are more likely to suffer from cachexia or more aggressive weight-loss is not known, but represents an interesting area for future exploration.

The potential biomarkers of cachexia in the current study were selected from evidence relating predominantly to muscle wasting in animal models. The lack of association of biomarkers with cachexia may therefore simply reflect differences between animal and human cancer cachexia. Whereas the majority of animal models of cachexia undergo rapid and profound weight-loss, human cancer cachexia is a chronic disease process. Furthermore, in humans, there will be added confounding factors such as level of baseline physical activity, bed rest, the presence of co-morbidities, dietary preferences, personal motivation and sickness behaviour.

The majority of potential biomarkers that were evaluated related to protein degradation rather than synthetic pathways. There is reasonable evidence to suggest that in muscle atrophy associated with ageing/ bed-rest, suppression of protein synthesis is of greater importance than increased degradation (34). Furthermore, in patients with UGIC, our group recently found evidence of suppression of muscle protein turnover (23). It may be that future studies investigating biomarkers selected from anabolic pathways may identify candidates which relate more strongly to cachexia. Alternatively, changes in muscle at the molecular level may not have a strong influence on muscle phenotype. Evidence of such dissociation has been demonstrated by Greenhaff et al in the skeletal muscle of healthy men. They showed that increased amino acid and insulin availability

led to changes in anabolic signalling molecules and components of the UPP which did not result in the expected corresponding alterations in muscle protein synthesis or breakdown (35). Whether this also occurs in the context of human cancer cachexia remains to be elucidated.

In conclusion, many of the key components of known muscle wasting pathways do not transpose directly to being robust biomarkers of cachexia. Skeletal muscle Akt protein levels/phosphorylation status and GABARAPL1 expression are biomarkers relating to cancer and possibly early cachexia. β-dystroglycan is a biomarker of weight-loss in cancer patients and MyHC and dystrophin are biomarkers associated with survival. This study highlights the complexity of biomarker research and provides impetus for further validation and discovery studies in order to identify robust diagnostic biomarkers and potential therapeutic targets in patients with cancer cachexia.

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Table 1 – Patient demographics for (**A**) HC and cancer patients (with and without cachexia) and (**B**) cancer patients surviving ≤ 1 vs > 1 year. Results are presented as mean ± SD or categorically except for median survival. Abbreviations: M, male; F, female; BMI, body mass index; CRP, C-reactive protein; KPS, Karnofsky performance score.

A.	**Control**	**All Cancer**	**Cancer No cachexia**	**Cancer Cachexia**
n=	15	92	41	51
M/F	8/7	66/26	34/7	32/19†
Age (yr)	56±17	65±10*	68±9	63±9†
Weight-loss (%)	0.0±0.0	8.1±9.3*	0.8±3.0	13.9±8.6†
Survival (days)	-	675	846	562†
BMI (kg/m^2)	28.0±4.5	25.7±4.0*	27.0±4.0	24.6±3.7†
CRP (mg/l)	3.5±2.7	15.5±31.3	12.0±29.6	18.3±32.6
CRP≥5mg/l (Y/N)	4/11	41/51	15/26	26/25
KPS	100±0	89±13*	92±10	86±14†

* = p<0.05 cancer vs control patients, † = p<0.05 cachectic vs non-cachectic patients

B.	**Survival ≤1yr**	**Survival>1yr**
n=	27	64
M/F	18/9	48/16
Age (yr)	66±10	65±10
Weight-loss (%)	12.0±11.1	6.3±8.0*
Survival (days)	245	1195*
BMI (kg/m^2)	25.3±3.5	26.0±4.2
CRP (mg/l)	18.0±36.3	14.6±29.4
CRP≥5mg/l (Y/N)	16/11	25/39
KPS	83±13	91±12*

* = p<0.05 survival >1yr vs survival ≤1yr

Table 2 – Patient demographics according to the ROC derived cut-offs for (**A**) MyHC and (**B**) Dystrophin. Results are presented as mean ± SD or categorically. Abbreviations: M, male; F, female; BMI, body mass index; CRP, C-reactive protein; KPS, Karnofsky performance score.

A. MyHC	**low (<0.87)**	**high (≥0.87)**
n=	12	35
M/F	7/5	21/14
Age (yr)	63±7	64±10
BMI (kg/m^2)	24.9±4.4	25.4±3.8
Weight-loss (%)	9.8±7.4	9.6±12.1
Cachexia (Y/N)	9/3	20/15
CRP (mg/l)	20.3±41.6	12.1±25.8
CRP≥5mg/l (Y/N)	7/5	14/21
KPS	88.2±10.8	92.1±12.1

B. Dystrophin	**low (<0.31)**	**high (≥0.31)**
n=	8	21
M/F	5/3	10/11
Age (yr)	62±13	63±9
BMI (kg/m^2)	26.8±6.3	25.0±4.3
Weight-loss (%)	9.5±7.5	9.7±11.3
Cachexia (Y/N)	7/5	14/21
CRP (mg/l)	45.8±52.3	11.8±22.4
CRP≥5mg/l (Y/N)	5/3	8/13
KPS	82.5±8.9	84.3±15.0

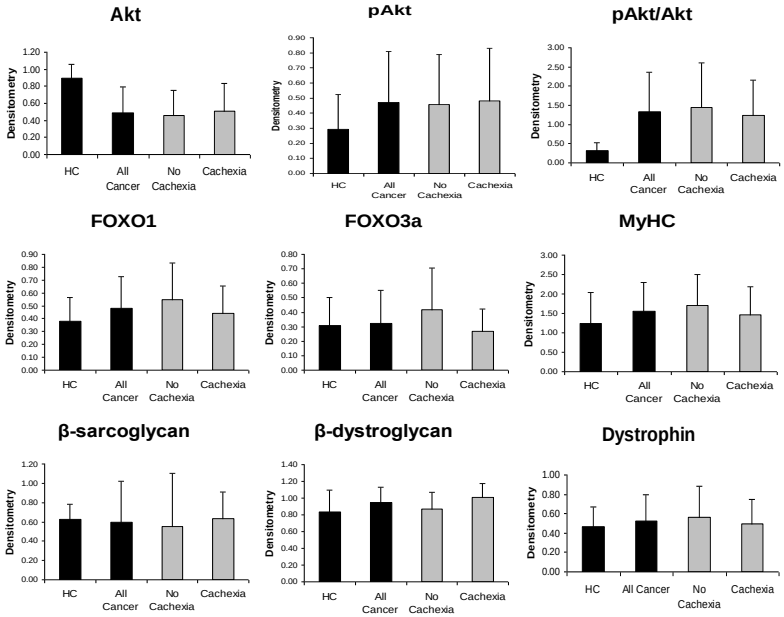
Figure Legends

Figure 1: Graphs showing (**A**) densitometry of protein biomarkers normalised to loading control or (**B**) delta-delta CT expression of mRNA biomarkers for HC and cancer patients (with and without cachexia). *$p<0.05$ HC vs all cancer, #$p<0.05$ no cachexia vs cachexia.

Figure 2: Kaplan-Meier survival curves for patients with (**A**) low (<0.87) vs high (≥0.87) MyHC protein levels; Log Rank p=0.023 and (**B**) low (<0.31) vs high (≥0.31) dystrophin protein levels; Log Rank p=0.008.

Figure 1:

A.


Akt
pAkt
pAkt/Akt
FOXO1
FOXO3a
MyHC
β-sarcoglycan
β-dystroglycan
Dystrophin
Densitometry
HC
All Cancer
No Cachexia
Cachexia

B.

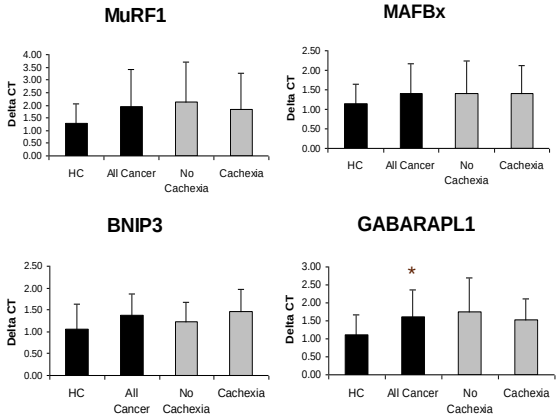
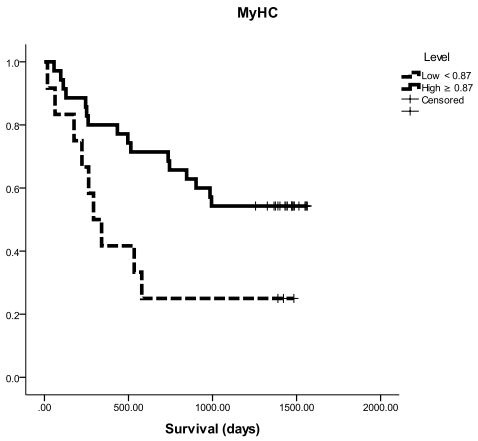
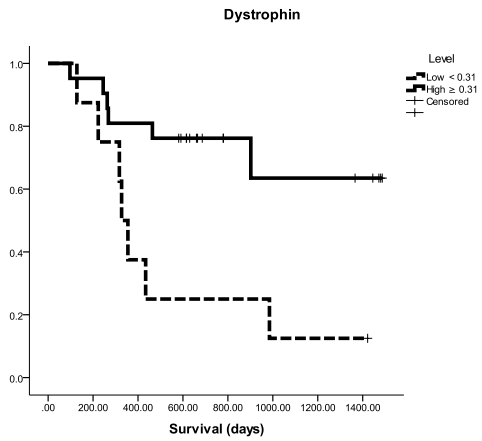

MuRF1
MAFBx
BNIP3
GABARAPL1
Delta CT
HC
All Cancer
No Cachexia
Cachexia
*

Figure 2:

A.


MyHC
Level
Low < 0.87
High ≥ 0.87
Censored
1.0
0.8
0.6
0.4
0.2
0.0
.00
500.00
1000.00
1500.00
2000.00
Survival (days)

B.


Dystrophin
Level
Low < 0.31
High ≥ 0.31
Censored
1.0
0.8
0.6
0.4
0.2
0.0
.00
200.00
400.00
600.00
800.00
1000.00
1200.00
1400.00
Survival (days)

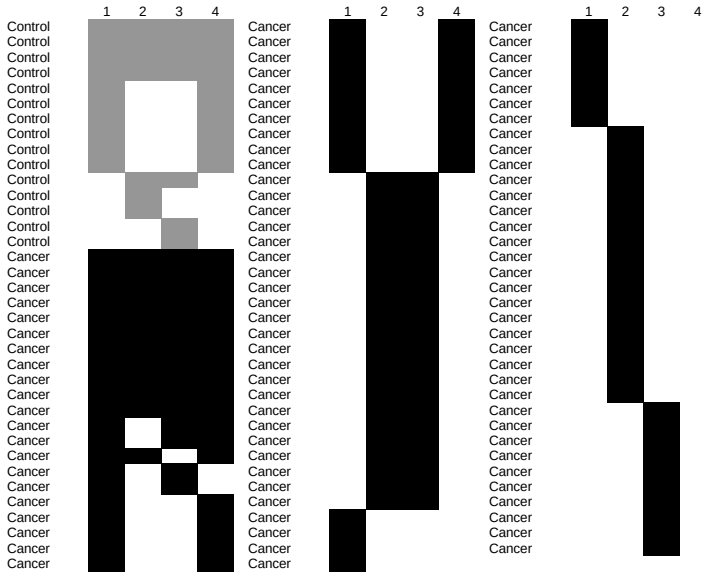
Supplementary Material

Supplementary Table 1
Individual cancer patient cohorts according to biomarkers evaluated. Abbreviations: M, male; F, female; BMI, body mass index; CRP, C-reactive protein; KPS, Karnofsky performance score.

	1	**2**	**3**	**4**
n=	42	52	47	29
M/F	28/14	34/18	28/19	15/14
Age (yr)	65±10	66±9	64±9	63±10
BMI (kg/m^2)	25.7±4.5	25.5±3.6	25.3±3.9	25.5±4.9
Weight-loss (%)	7.5±9.5	8.9±8.0	9.7±11.0	9.6±10.3
Cachexia (Y/N)	22/20	32/20	29/18	18/11
CRP (mg/l)	20.7±38.7	17.4±32.1	14.2±30.3	21.2±35.8
CRP≥5mg/l (Y/N)	20/22	26/26	21/26	23/16
KPS	83.1±13.7	92.4±10.5	91.0±11.7	83.8±13.5

1= Akt, pAkt, β-dystroglycan, β-sarcoglycan
2= FOXO1, FOXO3a, BNIP3, GABARAPL1, MuRF1, MAFBx
3= MyHC
4= Dystrophin

Supplementary Figure 1
Visual representation of the overlap in patient groups for different biomarkers (shaded box indicates that biopsy material was used).



1= Akt, pAkt, β-dystroglycan, β-sarcoglycan
2= FOXO1, FOXO3a, BNIP3, GABARAPL1, MuRF1, MAFBx
3= MyHC
4= Dystrophin